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| 14. ABSTRACT Expression in E.coli and purification of a prion protein peptide (residues 89-143 with a P101L mutation, designated P ₅₅), that has shown biological activity, has been done. Isotope labeling and solid state NMR with peptide produced in this was has been demonstrated, enabling further work to define the conformation of the infectious, fibrillar form. Fully C13/N15 labeled peptide was also produced and resonances in solution assigned to enable NMR detected hydrogen exchange of fibrils to be done. Mass spectroscopy detection of hydrogen exchange has also been pursued but conditions for fragmentation need to be further refined. The combination of solid state NMR and hydrogen exchange are being used to define conformations and interactions of the peptide backbone that will lead to a model for P ₅₅ in the fibril state. The kinetics of the fibrillization process of the P ₅₅ peptide have been studied to optimize conditions for fibril formation, and to enable comparison of initial small fibrils with larger mature ones. | | | | | |
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Table of Contents

| | |
|-----------------------------------|----|
| Cover..... | 1 |
| SF 298..... | 2 |
| Introduction..... | 4 |
| Body..... | 4 |
| Key Research Accomplishments..... | 10 |
| Reportable Outcomes..... | 10 |
| Conclusions..... | 10 |
| References..... | 11 |
| Appendices..... | |

INTRODUCTION

The goals of this project are to increase understanding of the conformation of infectious, fibrillar prion protein (PrP) to determine how this relates to the process of conversion of endogenous native prion protein from the cell. The methods being developed will also allow prion protein samples to be probe for conformational differences that lead to 'strain' behavior (different incubation times and distributions of PrP aggregates and neuronal loss in the brain) and may provide insights into the basis for such differences. Understanding these fundamental steps in the processes of initiation and propagation of the disease related state of the protein may lead to new approaches for preventing conversion to the pathogenic state. The experimental work as described in the original proposal applies two approaches, solid state NMR to determine specific conformational features of aggregated PrP related samples, and measurements of rates of hydrogen exchange for backbone amides in the sample to probe hydrogen bonding in aggregated states. The initial stages of the work are using the mouse peptide fragment containing residues 89-143 of PrP with the Pro101Leu mutation introduced, that we will refer to here as P₅₅ (for 55 residues). This peptide has been shown to induce prion disease when introduced into mice that express the P101L variant PrP [Kaneko *et al.*, 2000, Tremblay *et al.*, 2004]. It remains the simplest model for a protein that can induce prion disease. The specific approach being used is to prepare samples of P₅₅ from the mouse prion protein in new ways to facilitate isotope labeling for use in solid state NMR, and to carry out solid state NMR studies of the conformation of this peptide. We have done many experiments with *in vitro* translation, but have obtained consistently low yields (insufficient for solid state NMR) and hence have turned to expression in *E.coli* cells, with better results. We have also extended mass spectroscopic measurements of hydrogen exchange, but get limited coverage. To complement the MSDX approach we have also initiated solution NMR studies of dissolved fibril peptides to obtain individual amino acid resolution data (which will complement the MS data).

BODY

Production of PrP(89-143)P101L = P₅₅ samples

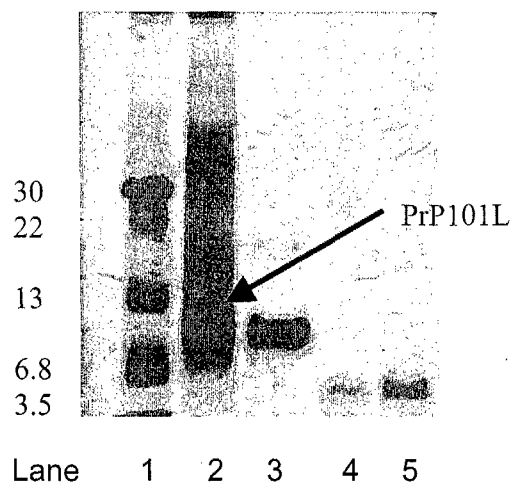
We continued efforts to produce peptides with *in vitro* translation using the Roche Rapid Translation system, based on *E.coli* cell lysates. The advantage of this approach is that enzymes that interconvert different amino acids are absent, this makes it possible to incorporate almost any isotope labeled amino acid into a protein without isotope scrambling whereas normal *in vivo* expression only allows less metabolically active amino acids to be labeled. We tried many variations of the basic protocol, trying to optimize plasmid purification and expression conditions. Experiments were done with an expression construct that encoded P₅₅ in the Roche optimized pIVEX 2.5d vector, including an N-terminal hexahistidine tag for purification (expression without this tag was not better), a tobacco etch virus protease cleavage site and then the P₅₅ sequence. Many controls were done including positive controls with a green fluorescent protein (GFP) vector that is known to express well. In spite of good performance with GFP only small, but measurable, amounts of P₅₅ were produced. Additional factors considered and tested were: loss of peptide through proteolysis by residual proteases in the translation lysate (no improvement was seen in the presence of protease inhibitors); distribution of the peptide to the outside 'feeding chamber' in the translation reactor (total yield

collecting both chambers gave no improvement); addition of detergents since some have improve translation yield for other proteins (no change in expression level was detected).



- 1 – P₅₅ expression no additive
 2 – P₅₅ expression added protease inhibitor cocktail (Roche)
 3 – P₅₅ expression added NDSB (a detergent)

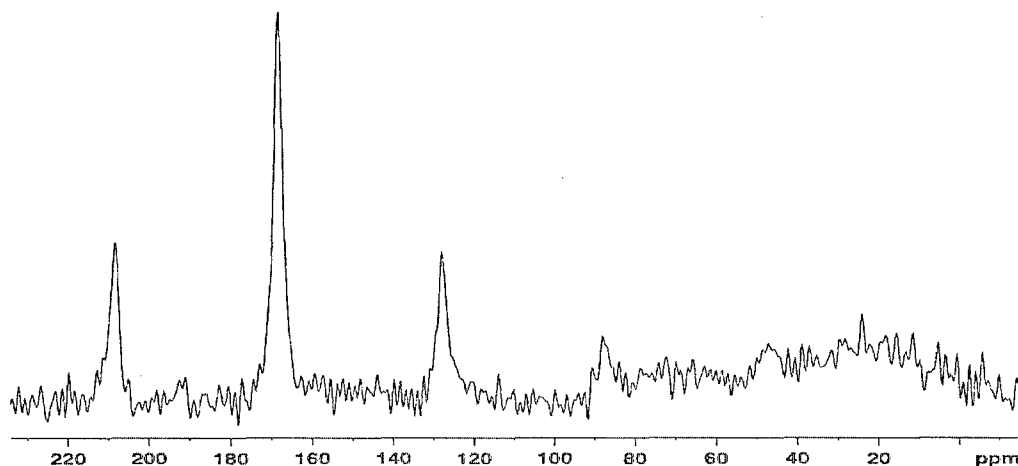
After a great deal of effort with the *in vitro* translation system we decided that it was critical to move on with solid state NMR and so have shifted to production of protein through expression in *E. coli* using the His tagged fusion described above. Initial expression yields were modest, and so expression was tested with a number of different strains of *E. coli*, including BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pACYC, with the latter giving the best expression. This strain expresses a repressor that gives very tight control of the expression plasmid until induction but then turns on strongly. The better performance with this strain suggests that the *E. coli* are adversely affected by the presence of the expressed peptide. We also tested sugar based autoinduction system (with defined growth medium) developed by W. Studier [Studier, 2005], which also has a tight control system. Both worked well and under optimized conditions we have been able to get ~8 mg / L of peptide with added labeled amino acid.



Lane 1, protein m.w. standards; Lane 2, His tagged P₅₅ in *E. coli* lysate (1 L growth); Lane 3, Ni column purified P₅₅; Lane 4, TEV cleaved P₅₅ removing His tag; Lane 5, cleavage product after repurification to remove His tag.

This method was used to prepare ¹³C/¹⁵N labeled protein required to do the assignments for the solution NMR / hydrogen exchange that was described above, and to prepare

uniformly ^{15}N labeled protein for doing the actual exchange experiments on fibrils. The production protocol seems to be reproducible and reasonably efficient. For solid state NMR peptide labeled with ^{13}C at the carboxyl of methionines was made. The cleaved peptide product was fibrillized (kinetic studies discussed below), and the first solid state NMR data have been collected.



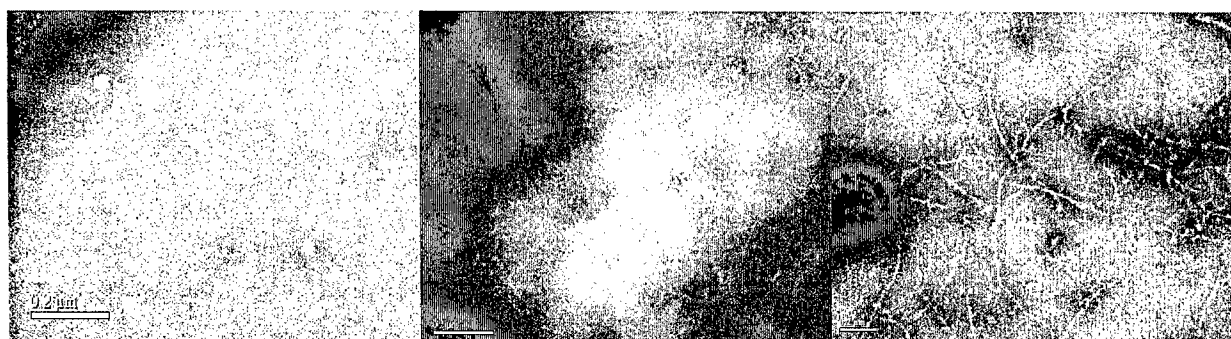
^{13}C magic angle spinning spectrum of P_{55} labeled on methionine carbonyls, peptide was fibrillized and then lyophilized. The observed linewidth is about 3 ppm (comparable to previous samples of this peptide prepared synthetically), the chemical shift is consistent with beta conformation though since only one line is seen (probably degenerate) we must be cautious in interpretation.

The next samples that will be prepared will be labeled (through expression) at leucine residues (4 in the P_{55} sequence, two before and two after the most studied region of residues 112-124), we anticipate we may see an equilibrium between ordered and disordered residues based on recent experiments. We will also make samples with labeled histidine (3 in P_{55}) and tyrosine / tryptophan (1 each in P_{55}). Further labeling sites will be chosen based in the results from these experiments. We will also compare linewidths in lyophilized sample to those from wet fibrils spun into sample spinning rotors. Our past results from solid state NMR were written into a manuscript submitted for publication.

Fibrillization of peptide samples

In our work we have prepared samples in the same manner that Prusiner's lab originally did since the activity of fibrils prepared under such conditions has been established. The samples are allowed to stand in solutions mixed buffer and acetonitrile, 4°C for three weeks. In order to determine what changes in the samples occur over this time period, how variation in the process affects the product, and possibly to speed production of samples we carried out a series of measurements of the process of fibrilization of P_{55} peptide as a function of time and conditions (somewhat analogous to fibril growth analysis on $\text{A}\beta$ peptide). A series of samples was prepared at initial peptide concentrations of 1, 5 and 10 mM concentration, and these were incubated at 4, 25 and

37°C. Aliquots were taken (more frequently near the start), centrifuged to pellet any fibrils formed, and then the absorbance of the remaining soluble material at 280 nm was determined and converted to concentration. We also determined the amount of light scattering at 600 nm (at which there is no real absorbance). Samples of the pelleted material were examined by electron microscopy to determine whether there were changes in morphology. We find that most of the peptide is already in aggregates at 24 hours for all peptide concentrations, somewhat more slowly at lower temperature but not dramatically so. Light scattering increases due to fibril formation but then decreases somewhat, suggesting that there are fewer, larger scattering aggregates as time goes on. This behavior shows in the electron microscopy data as well, even at the time aggregation is (nominally) initiated (by addition of acetonitrile) there are fibrils seen in the electron micrographs. However with increasing incubation time larger, better developed fibrils can be seen (examples of micrographs are shown below). This parallels processes of protofibril formation followed by fibril formation seen with other peptides [Cannon *et al.*, 2004].



Buffer Only

0 hours
(immediately following making
the fibrillization sample)

1 Week

We will continue studies of 'matured' fibrils, those left in the fibrillization conditions for >2 weeks, for the initial solid state NMR studies. However with some samples we will prepare samples from material taken at short times after initialization of fibril formation to see if there are spectroscopic differences that can be seen. If there are we want to define regions that are sensitive to the change in fibril morphology to determine what processes might be occurring and how they might relate to the *in vivo* activity of the peptide.

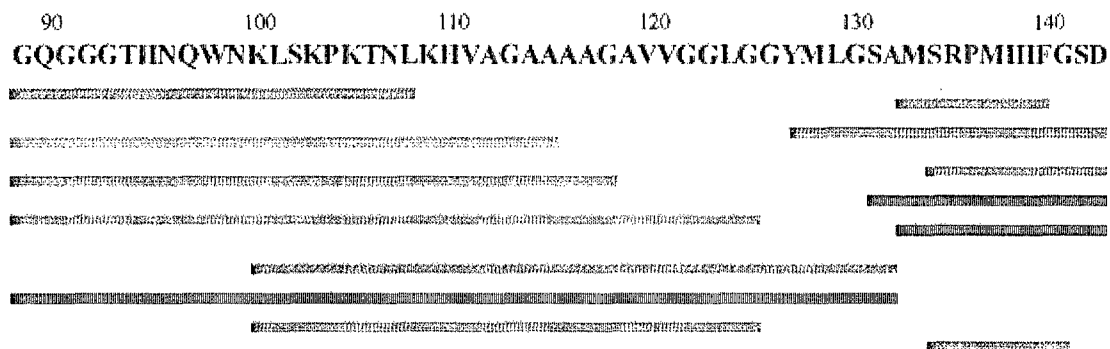
MALDI-MS hydrogen-exchange experiments

PrP fibrils that were prepared in normal H₂O were incubated in deuterated 20 mM potassium phosphate buffer, pD 7.4. Under these conditions deuterium exchanges relatively rapidly into the amide groups of amino acids for which the amide is exposed to solvent, but not into those that are hydrogen bonded. After 5 minutes of in-exchange, aliquots were centrifuged for 1 minute at 8,000 G to collect the fibrils, and the supernatant decanted. Fibrils were rapidly dissolved at 0 °C in 8% TFA (trifluoroacetic acid), followed by a ten fold dilution with water to 0.8 % TFA (conditions that quench

exchange). The solubilized peptide was then digested with immobilized pepsin (Pierce) at 0 °C for 10 min. An aliquot was then removed and centrifuged at 1000 G for 30 seconds and the supernatant removed. Each aliquot was diluted 5 fold prior to MALDI mass spectral analysis.

We had originally identified 14 peptic peptides that afforded 100% sequence coverage of the PrP peptide, upon rapid solubilization under quenching conditions. Incorporation of deuterium into the peptide leads to broadening of the isotopic envelope and a decrease in signal to noise. Preliminary hydrogen exchange experiments revealed that only 3 of the previously identified peptides provided sufficient signal to noise to be useful for monitoring hydrogen exchange (Figure below). One peptide corresponds to the first 44 amino acids (residues 89-132), and the other 2 are short fragments of the C-terminus (residues 133-143 and 131-143). Unfortunately, this set of peptides limits localization of deuterium incorporation.

The low signal to noise most likely results from the inability of pepsin to cleave the PrP sequence (rather than from differences in ionization efficiency). We attempted to increase peptic fragmentation by vary the concentration of PrP peptide, pesin resin, and length of digestion with little success. Based on the fragments produced, it appears that pepsin can only easily cleave at the S131-A132 and A132-M133 bonds. This could be either due to the intrinsic sequence preferences of pepsin or due to local structural conformations of the PrP peptide. We can attempt to address these issues by adding chemical denaturants such as urea or guanidine hydrochloride to the fibril dissolution buffer, or by using a second protease such as fungal protease XIII. Both these methods have been used by Virgil Woods' lab to optimize peptide fragmentation for hydrogen exchange mass spectrometry measurements.



Peptides identified from peptic digest. Gray bars indicate peptides with significant signal to noise loss upon deuteration. Blue bars indicate peptides with sufficient signal to noise.

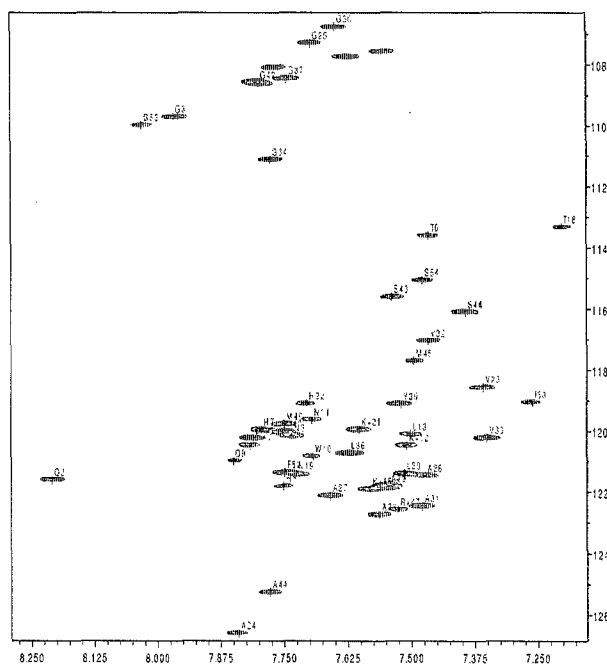
NMR based hydrogen-exchange experiments.

In order to provide higher sequence resolution information on the exchange properties of residue in P₅₅ we considered using a NMR based measurement of hydrogen exchange. PrP fibrils can be rapidly dissolved in acidic DMSO, which also serves to quench the hydrogen exchange. The incorporation of deuterium can be monitored by collecting a

^{15}N - ^1H HSQC spectra, a two dimensional experiment that correlates bonded proton and nitrogen pairs, producing a single cross peak for each amino acid. Peaks disappear when the ^1H is replaced by ^2H through exchange. This experiment requires the sample to be uniformly enriched with ^{15}N , and provides hydrogen exchange data at amino acid.

Prior to starting these NMR based hydrogen exchange experiments, all of the cross peaks in the ^{15}N HSQC spectrum needed be associated with the specific amino acid in the sequence from which they derive. We accomplished this using NMR assignment strategies on a uniformly isotope enriched ^{13}C , ^{15}N P₅₅ sample that was generated by expression in E.coli as discussed above. Below an ^{15}N HSQC spectrum of PrP peptide (concentration = 150 μM) in 95% d₆-DMSO, 5% H₂O, pH 5 is shown. Cross peaks are present for all amino acids except the prolines and N-terminal glycine (proline has no amide and the N-terminus exchanges rapidly). NMR assignments were made using HNCA, HNCACB, and CBCA(CO)NH three dimensional, triple resonance experiments. All residues are unambiguously assigned save for gly-4, gly-5, gly-35, and gly-38, which are found as gly-gly pairs in the sequence. Due to the degenerate C α chemical shifts of these residues, they cannot be distinguished from each other. They can however be discerned based on their nitrogen chemical shifts using a HCAN experiment. Collection of these data is in progress, absence of these assignments does not slow the analysis of the other residues.

^{15}N HSQC spectrum of P₅₅ peptide in DMSO. Peak assignments are indicated with the residue label and number of the amino acid giving rise to the peak.



Samples of P₅₅ labeled just with ^{15}N have been made for doing the hydrogen exchange experiments.

The first exchange experiment used a small amount of peptide, and fibrils that had not been fully matured. A good deal of the fibrillar material dissolved during the exchange, and there was not a sufficient amount left for NMR analysis. New samples are being prepared now, fully matured fibrils will be used in greater quantity in the next experiments to avoid the problems that we encountered.

KEY RESEARCH ACCOMPLISHMENTS

- completed trials of *in vitro* expression of P₅₅ but could only obtain low yields
- established expression of P₅₅ in *E.coli* with good yield, established purification protocol
- made ¹³C-methionine labeled P₅₅ and collected solid state NMR data, uniformly C/N labeled P₅₅ for solution NMR
- determined fibrillization kinetics of P₅₅ and studied fibrils formed by electron microscopy
- assigned resonances of DMSO dissolved P₅₅ for probing hydrogen exchange
- established conditions for doing hydrogen exchange on fibrils with NMR detection

REPORTABLE OUTCOMES

Lim, K.-H., Nguyen, T.N., Damo, S.M., Mazur, T., Ball, H.L., Prusiner, S.B., Pines, A. and Wemmer, D.E. "Solid State NMR Structural Studies of the Fibril form of a Mutant Mouse Prion Peptide PrP₈₉₋₁₄₃(P101L)" submitted to Solid State NMR.

CONCLUSIONS

We have established expression of tagged P₅₅ peptide in *E.coli* for production of isotope labeled peptide, and worked out purification protocols. Solid state NMR spectra have been collected for the first sample prepared this way and are of good quality indicating that we can now proceed with other labeling patterns. The inability to us *in vitro* translation is disappointing as our choice of amino acids to label is somewhat more limited, however with the combination of methods being used this should not limit our ability to study the system substantially. The solid state NMR studies will define the conformation of the P₅₅ backbone. Fibrils are comprised of regions of peptide that are primarily beta sheet but can also have turns. The aim of our experiments are to determine which amino acids are in beta-sheets, turns, or disordered regions. This will allow development of a more detailed model of the fibril structure which will help with understanding the processes leading from native protein to infectious fibrils. Being able to define regions involved in various structures also means that we will be able to detect changes in such structure, as anticipated in different prion strains. We have also examined the process of fibrillization of the P₅₅ peptide, and hence can now more rationally choose conditions for preparation of fibrils for both the solid state NMR and hydrogen exchange measurements.

We continued mass spectroscopy based studies of hydrogen-deuterium exchange into fibrils of the P₅₅ peptide. However we found that the broadening of the isotope distributions, which weakens the mass spectrum signal for each particular combination of peptide and extent of exchange, lead to a loss of signal to noise ratio so that quantification was problematic. This can be addressed by either addition of denaturant to increase the extent of proteolysis, and switching to or adding another protease, again to increase the amount of fragmentation. Since we want to establish a baseline at single amino acid resolution, we initiated NMR detected studies of hydrogen exchange. The first, somewhat work intensive stage of this is to assign resonances under conditions of solubilization of fibrils and quenching of exchange. Following work with other peptides we found conditions with dimethylsulfoxide solvent with trifluoroacetic acid and a small amount of water that allow fibrils to be quickly dissolved and provide good conditions for NMR. Using expressed, doubly ¹³C/¹⁵N labeled peptide backbone resonances were nearly

completely assigned, and hydrogen exchange measurements can be started. These measurements will establish which amino acids are involved in hydrogen bonds in fibrils, complementing the solid state NMR data. However the hydrogen exchange approach can be extended to small amounts of protein (including natural PrP from cells) to tie together the results from *in vitro* studies of peptides with the real behavior of PrP *in vivo*. This will enable analysis of naturally occurring strains of prions in the long term.

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